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Amino Acid- and Purine Ribonucleoside-Induced Germination of *Bacillus anthracis* ΔSterne Endospores: *gerS* Mediates Responses to Aromatic Ring Structures

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Specific combinations of amino acids or purine ribonucleosides and amino acids are required for efficient germination of endospores of *Bacillus anthracis* ΔSterne, a plasmidless strain, at ligand concentrations in the low-micromolar range. The amino acid L-alanine was the only independent germinant in *B. anthracis* and then only at concentrations of >10 mM. Inosine and L-alanine both play major roles as cogerminants with several other amino acids acting as efficient cogerminants (His, Pro, Trp, and Tyr combining with L-alanine and Ala, Cys, His, Met, Phe, Pro, Ser, Trp, Tyr, and Val combining with inosine). An ortholog to the *B. subtilis* tricistronic germination receptor operon *gerA* was located on the *B. anthracis* chromosome and named *gerS*. Disruption of *gerS* completely eliminated the ability of *B. anthracis* endospores to respond to amino-acid and inosine-dependent germination responses. The *gerS* mutation also produced a significant microlag in the aromatic-amino-acid-enhanced-alanine germination pathways. The *gerS* disruption appeared to specifically affect use of aromatic chemicals as cogerminants with alanine and inosine. We conclude that efficient germination of *B. anthracis* endospores requires multipartite signals and that *gerS*-encoded proteins act as an aromatic-responsive germination receptor.

Bacillus anthracis, like other members of the genus *Bacillus*, produces a dormant morphotype, the endospore, in response to adverse environmental conditions. Endospores are resistant to physical and/or chemical insults and are capable of protracted dormancy (2). As the infective particle for the initiation of anthrax is the endospore, it is important to identify the signals that trigger breakdown of endospore dormancy.

Germination occurs when bacterial endospores break dormancy and return to vegetative growth. Small molecules, germinants, whose identities vary between *Bacillus* species, trigger this process. Three well-studied examples are the response to L-alanine in *Bacillus subtilis*, L-proline in *Bacillus megaterium*, and inosine in *Bacillus cereus* (1, 10, 16, 17, 22). The present model is that germinants bind to membrane-associated protein receptors encoded by tricistronic operons typified by the *gerA* operon of *B. subtilis* (2, 14, 19). The *gerA* family of operons is found throughout the *Bacillus* and *Clostridium* genera, and mutations in these operons have been shown to cause loss of germination responses to specific germinants (11, 13, 14). Through an unclear mechanism, this binding event leads to the breakdown of spore dormancy and a return to vegetative growth.

The germination of *B. anthracis* endospores has been studied sporadically over the past 60 years. In 1949, Hills showed that germination was influenced by L-alanine, tyrosine, and adenosine (7). This pioneering work was unable to strictly determine which of these germinants acts independently or in concert with the others. Work by Titball and Manchee showed L-alanine initiating the germination of the *B. anthracis* endospore. Their work went further in showing, as in *B. subtilis*, that the L-alanine germination activity in *B. anthracis* was inhibited by D-alanine (21). Strict delineation of the defined germinants for *B. anthracis* is therefore still lacking, as is attribution of germination phenotypes to specific *gerA*-like operons in *B. anthracis*. The recent discovery and elucidation of a *gerA*-type operon, *gerX*, on the pXO1 virulence plasmid, whose deletion somewhat attenuates *B. anthracis* in a mouse model (5), indicated that germination plays a role in *B. anthracis* pathogenicity.

We now explore the role of specific amino acids and purine ribonucleosides in initiating germination individually and in concert with each other. A plasmidless strain of *B. anthracis* was utilized to determine the chromosomally controlled germination signals. We present evidence for multiple germination responses and a mutation in one chromosomal locus, *gerS*, which eliminates two of these germination responses, both of which include a role for aromatic ring structures. In addition, these data support a hypothesis that multiple signals and most likely multiple *gerA*-type operons are required to break dormancy in *B. anthracis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The parental strain of *B. anthracis* used is ΔSterne 34F2. The strain is a plasmidless variant, derived from the Sterne 34F2 strain by temperature curing of the pXO1 plasmid, and was used here to better identify chromosomally encoded phenotypes. *B. anthracis* ΔSterne was grown in brain heart infusion broth (BHI; Difco) with rapid agitation. Selection of *B. anthracis* transformants and crossover events was done with chloramphenicol (15 μg/ml) or erythromycin (1 μg/ml). *Escherichia coli* was manipulated and stored according to accepted protocols. All growth of *E. coli* was in either BHI or L broth (Difco). Ampicillin resistance was selected at 100 μg/ml. Colonies were grown on solid media with 15 g of Bacto-agar (Difco)/liter added to either

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BHI or to L broth. Strains were stored long term at −70°C in BHI with 20% glycerol.

Construction of *B. anthracis* ΔSterne 34F2 Δ*gerSA* strain. The putative *gerS* operon lies on contig no. 4752 of the publicly available The Institute for Genomic Research (TIGR) sequence for *B. anthracis* (unfinished; preliminary sequence data was obtained from TIGR's website at http://www.tigr.org). The TIGR sequences used to make the construct are based on an Ames-based *B. anthracis* strain. However, primers from this sequence amplified a fragment from our genome of identical size to the predicted Ames sequence, and the restriction pattern of the resultant fragment was identical to that predicted from Ames (data not shown). These observations are consistent with the previously reported low heterogeneity in the *B. anthracis* genomic cluster (9, 18). The putative *gerSA* open reading frame (ORF) begins at bp 18457 of contig no. 4752. A fragment overlapping the beginning of *gerSA* and a putative σ^G promoter (bp 19289 to 17691 on contig no. 3924) (12) was amplified from *B. anthracis* ΔSterne 34F2 genomic DNA by PCR (forward primer, GGC GTT AAG CTT CCC AGT CTA TTG; and reverse primer, GCT GTT GTG TCA GGG AAT TCA GTT ATT G). Using engineered *Eco*RI and *Hin*dIII sites in the primers, the fragment was cloned into pT7Blue (Novagen). The cloned fragment was truncated by inverse PCR of the cloned fragment with engineered *Bam*HI sites and religation (forward primer, AAG CGG ATC CAA AAA GAA GTA TT; and reverse primer, TGA ACC GAA AGT GGA TCC AAG TGA). The truncated fragment had lost the putative promoter and start of the *gerSA* ORF. An erythromycin cassette (4) was inserted into the *Bam*HI site, and the resultant suicide vector was transformed in *B. anthracis* ΔSterne 34F2. Recovered Ery^r colonies were screened by PCR and Southern blotting to confirm a single crossover event into the chromosome. The transformation and integration were done successfully in three separate trials. Every attempt resulted in identical phenotypes among the recovered clones, confirming that the phenotype reported is linked to the deletion of the GerSA coding region. Since the layout of the putative *gerS* operon is consistent with studied *gerA*-type operons and the resistance cassette is polar for the downstream ORFs, it is reasonable to assume that the function of the entire *gerS* locus is inactivated by the insertion in front of the putative *gerSA*, as with similar systems.

Endospore preparation. *B. anthracis* endospores were prepared by growing an overnight culture (16 h at 37°C with vigorous shaking) in BHI with 0.5% glycerol from a single colony off solid media. The overnight culture was diluted 1:10 into fresh CCY media and was then grown with vigorous shaking for 24 to 48 h (1). Cultures that were >90% refractile endospores, as determined by phase-contrast microscopy, were centrifuged for 30 min at 1,500 × *g*. The resulting pellet was resuspended and vigorously washed three times with sterile distilled water, and pellets were collected by centrifugation. After the final wash, bacterial pellets were resuspended in 1.0 ml of sterile distilled water and heat treated at 65°C for 30 min. Endospore suspensions were washed and centrifuged four times, with the uppermost layer of the pellet being discarded at each wash. Cleansed endospore preparations were checked by phase-contrast microscopy to assure >95% refractile endospore bodies and an absence of gross contamination with vegetative debris. Cleaned endospore preparations were resuspended in sterile distilled water and were stored at room temperature. Storage conditions were not seen to alter the observed phenotypes. These procedure are similar to those used to produce endospores for virulence studies and as such do not appear to change the properties of the endospores from those used to experimentally infect animal models (6, 8).

Calcium release germination assay. During sporulation, very large amounts of calcium are incorporated into the endospore core where it chelates with dipicolinic acid. Release of calcium ions from the endospore into the environment is an early postcommitment stage of germination and is directly proportional to the number of actively outgrowing bacilli in rich media (20). Sporulating *B. anthracis* cultures in the presence of radioactive calcium (^{45}Ca) provides a marker for detection of early germination events. These radiolabeled endospores are germinated, and the percent germination of the population is determined by the free radioactive calcium in solution (20). Major advantages to this technique include the rapid and direct measurement of germination, the precision allowed by a high signal-to-noise ratio, and the ability to quantitate the number of germinating bacteria in a complex sample (i.e., biological samples). Radioactive calcium labeling in no way interfered with endospore formation, viability, or germination. *B. anthracis* endospores were prepared as described above, with the exception that 1 μCi of $^{45}CaCl_2$ (ICN Radiochemicals)/ml was added to the CCY medium. Radiolabeled endospores were mixed with germinant solution (routinely 10^6 endospores/ml), and samples were taken at regular time points (as indicated) and were then passed through a 0.22-μm-pore-size syringe filter. A typical specific activity is 10^5 cpm per 10^6 endospores. The filtrate was mixed 1:10 with Safety-Solve (Research Products International) and was analyzed in a scintillation counter (Beckman Instruments) with a standard ^{45}Ca window for 0.5 min. Samples were analyzed in at least duplicate on at least two independent endospore preparations. Percent germination is equivalent to percent ^{45}Ca released into the medium. The percent calcium released is calculated by the following formula: $Y = [(X - C)/(M - C)] \times 100$, where Y is the percent ^{45}Ca released (equivalent to percent germination), X is the radioactivity detected in the filtered sample of the germination reaction at a given time (experimental radioactivity, counts per minute), C is the radioactivity detected in a filtered sample of the germination reaction at time zero (background radioactivity, routinely under 100 cpm), and M is the radioactivity detected in an unfiltered sample of the germination reaction (maximum radioactivity, around 2,000 to 8,000 cpm/sample).

Germination reaction conditions. There are many variables affecting the germination rate (G_i) of endospores. In order to narrow the interpretation of results to those concerning the germinants tested, we describe a specific defined basal germination medium and a set of baseline conditions with which to test for germination responses. Since *B. anthracis* is a pathogen, a simple basal medium for the germination assays that approaches some of the major components of physiological fluids was tested. A sodium phosphate solution (10 mM NaH_2PO_4) with 100 mM sodium chloride (NaCl) is the basal medium. The basal medium was adjusted to a pH of 7.2 ± 0.2 by the addition of sodium hydroxide (NaOH) or hydrochloric acid (HCl) after the addition of germinants and remained stable throughout all experiments. All reactions were done at room temperature (22 ± 3°C), but no significant differences were seen at 37°C for several assays. The pH of the reaction was kept at 7.2 ± 0.2 for all reactions when the role of pH was not directly addressed.

Kinetic analysis. The G_i used for this work is the initial G_i, defined as the slope of the initial linear portion of the percent germination versus time plot. This rate is the maximum rate for a given set of conditions. For surveys of a large number of germination conditions, the initial G_i can be estimated to a first approximation by taking a 10-min stop point measurement of percent germination. Single-point measures show decisive on/off phenotypes, while more definitive time courses are used to assign actual rates. All kinetic measurements were done at least in duplicate on at least two independent endospore preparations.

Kinetic assays were done for a maximum of 21 min, though in most cases for 10 min (with 2-min time points). Empirical work with controls showed that the majority of strong, rapid germination reactions occur within the first 15 min and that the linear portion of the reaction was contained in the first 10 min. Samples that did not germinate in the initial time course were checked for delayed germination by looking at 30-, 60-, and 90-min points. Samples were also incubated for 16 h to detect extremely delayed germination; detection of germination after 16 h was not evaluated. Complete loss of a germination phenotype was defined as no detectable germination after more than 16 h.

RESULTS

Justification of *B. anthracis* strain and sporulation conditions. *B. anthracis* ΔSterne 34F2, a plasmidless derivative of the *B. anthracis* Sterne strain, a commonly used model strain, was chosen in light of two facts. First, both plasmidless strains of *B. anthracis*, as well as pXO1-deficient strains bearing only the pXO2 virulence plasmid, have been shown to germinate and outgrow in animal models (24). Second, in a previous study with the *gerX* locus, in vivo germination was observed, albeit at a somewhat lower rate (5). Both of these facts lead to the conclusion that in vivo germination is not solely under the control of the pXO1 plasmid or the *gerX* locus in particular. We pursued studies utilizing a plasmidless strain to limit the influence of the pXO1 plasmid and to determine what chromosomally encoded phenotypes and loci were present and relevant in *B. anthracis*. A potential concern is whether laboratory-produced endospores differ in their properties from endospores obtained from animal infection. While this exists as a formal possibility, it is likely that the two are equivalent, since multiple studies have shown that laboratory-produced endospores do in fact cause a seemingly natural infection in all animal models tested (6, 8), and work done with the *gerX* locus suggests that any change in the overall germination phenotype

TABLE 1. Germination survey for *B. anthracis* of major naturally utilized amino acids with subgerminal L-alanine[c]

Germinant[a,d]	% Germination in 15 min[b] for:	
	Parental strain	Δ*gerS* strain
Buffer control	3.4 ± 0.4	4.5 ± 1.3
Alanine (1 mM)	5.6 ± 1.8	3.4 ± 1.2
Alanine (100 mM)	98.0 ± 4.8	75.0 ± 2.3
Alanine/histidine	31.0 ± 1.5	2.1 ± 0.3
Alanine/proline	56.1 ± 4.6	37.6 ± 3.9
Alanine/tryptophan (1 mM)	49.4 ± 5.2	3.7 ± 0.3
Alanine/tyrosine (1 mM)	56.1 ± 6.4	3.7 ± 0.7

[a] Alanine is at 1 mM, unless otherwise noted. All secondary amino acids are L-isomers at 100 mM, unless otherwise noted. No amino acid other than alanine caused germination independently.
[b] Results are the average of triplicate experiments on two independent preparations with 1 standard deviation.
[c] An alanine concentration of 1 mM is considered subgerminal.
[d] The following amino acids were tested (100 mM each) in conjunction with alanine (1 mM) but were found not to alter the percent germination significantly above the alanine (1 mM) baseline: arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, and valine.

TABLE 2. Germination survey for *B. anthracis* of major naturally utilized amino acids with inosine

Germinant[a,c]	% Germination in 15 min[b] for:	
	Parental strain	Δ*gerS* strain
Inosine	5.6 ± 1.3	4.5 ± 1.3
Inosine/alanine	73.1 ± 1.5	51.0 ± 6.7
Inosine/cysteine	46.3 ± 1.9	2.7 ± 1.8
Inosine/histidine	64.4 ± 4.1	1.9 ± 0.8
Inosine/methionine	77.0 ± 6.3	2.3 ± 1.0
Inosine/phenylalanine	74.4 ± 4.0	2.4 ± 0.6
Inosine/proline	72.6 ± 3.0	2.5 ± 1.2
Inosine/serine	84.5 ± 8.6	3.7 ± 0.3
Inosine/tryptophan (1 mM)	79.1 ± 2.9	2.8 ± 0.7
Inosine/tyrosine (1 mM)	74.1 ± 2.1	2.1 ± 0.5
Inosine/valine	62.4 ± 4.3	2.2 ± 0.8

[a] All amino acids are L-isomers at 100 mM, unless otherwise noted. Inosine is at 1 mM.
[b] Results are the average of triplicate experiments on two independent preparations with 1 standard deviation.
[c] The following amino acids were tested (100 mM each) in conjunction with inosine (1 mM) but were found not to alter the percent germination significantly above the inosine (1 mM) baseline: arginine, asparagine, aspartate, glutamate, glutamine, glycine, isoleucine, leucine, lysine, and threonine.

might be reflected by significant changes in the virulence of the endospores.

L-Alanine-induced germination. This work focuses on the role of amino acids and ribonucleosides on the germination of *B. anthracis*. While there are a number of other chemical families that could be investigated for germination signals, these two have been of particular relevance in the *Bacillus* genus (1, 3, 7, 14–16). A significant germination response to high concentrations (100 mM) of the amino acid L-alanine is observed (Table 1), supporting the earlier work of Titball and Manchee (21). Virtually no interference from putative alanine racemase activity was observed, negating the need for an alanine racemase inhibitor (such as *O*-carbamyl D-serine) to attain rapid germination. The germination kinetics of alanine were extremely rapid; at 100 mM L-alanine in 10 mM NaH_2PO_4, over 90% of the endospores germinated in less than 10 min. A graph of G_i versus G_i/[alanine] was not linear, indicating that the reaction is complex and not easily defined as a simple binding event of alanine to a single alanine trigger (data not shown).

Germinant surveys with L-alanine as cogerminant. Though physiological levels of L-alanine (~1 mM) are subgerminal in *B. anthracis* (Table 1), they may potentiate other chemicals in triggering a robust germination response (1, 23). This is observed even when the second germinant stimulates no germination on its own at any concentration. All major naturally utilized amino acids were tested for the ability to trigger germination alone or in combination with each other at a high concentration (100 mM or as indicated). No significant germination was observed in the absence of alanine for any secondary amino acid or combination of them within the first 4 h (data not shown). In the presence of 1 mM L-alanine, four amino acids were found to trigger significant and rapid germination: L-histidine, L-proline, L-tryptophan, and L-tyrosine (Table 1). The alanine/tyrosine reaction was evaluated, and logarithmic concentration dependence was found for both tyrosine and alanine (0.001 to 1 mM range for each amino acid, data not shown). Under extended incubation (16 h) and at high concentration (100 mM), three amino acids could trigger an independent response: L-alanine, L-cysteine, and L-serine, with the latter two stimulating with only very slow kinetics (approximately 10 to 25% total germination in 16 h). When the remaining 17 naturally occurring amino acids (100 mM each or, as indicated, minus alanine, cysteine, and serine) were tested as a group for 16 h, no germination was detected (data not shown). Though we cannot totally discount the possibility of minute contaminants in our solutions, this potential problem is minimized experimentally by use of multiple stock sources, multiple lots, and multiple vendors. These data support a role for subgerminal L-alanine as an important cogerminant but only in concert with a small subset of the other amino acids.

AAID germination. Studies with *B. cereus*, a close genetic relative of *B. anthracis*, have shown that inosine and purine ribonucleosides in general are potent independent germinants (1). In *B. anthracis*, inosine alone (up to 50 mM) did not trigger an independent germination response in our experimentation, even when incubated for 16 h (data not shown). When the major naturally utilized amino acids were surveyed with inosine (1 mM), numerous strong germination responses were detected (Table 2). Strong germination responses are defined as those causing 40% or greater germination in 15 min. There remained detectable when the amino acid component concentration was lowered by 1 order of magnitude (10 mM for most amino acids and 0.1 mM for L-tyrosine and L-tryptophan). Those combinations that did not meet the requirements of a strong reaction were not pursued further. Strong responses were governed by the following amino acids in the presence of 1 mM inosine: L-alanine, L-cysteine, L-histidine, L-methionine, L-phenylalanine, L-proline, L-serine, L-tryptophan, L-tyrosine, and L-valine (Table 2). As a prototype of inosine/amino acid interactions, the serine/inosine combination was tested, and logarithmic concentration dependence was found for each component (0.001 to 10 mM range for each component, data not shown). By using L-serine as an amino acid control (1 mM), numerous ribonucleosides and ribonucleotides were then

TABLE 3. Effect of *gerS* mutation on high-concentration solution kinetics in *B. anthracis* ΔSterne 34F2

Germinant[b]	Maximum G_i[a] (% germination/min)		Score for 16-h germination[c]	
	Parental	Δ*gerS*	Parental	Δ*gerS*
Alanine	6.1 ± 0.4	7.3 ± 0.9	+	+
Alanine/histidine	6.7 ± 1.8	6.2 ± 0.5	+	+
Alanine/proline	9.1 ± 2.1	7.2 ± 0.7	+	+
Alanine/tryptophan (1 mM)	11.0 ± 1.5	6.3 ± 0.5	+	+
Alanine/tyrosine (1 mM)	10.1 ± 1.1	7.4 ± 0.6	+	+
Inosine	<0.1	<0.1	−	−
Inosine/alanine	16.5 ± 1.9	7.2 ± 1.0	+	+
Inosine/cysteine	10.0 ± 0.3	<0.1	+	+
Inosine/histidine	3.5 ± 1.1	<0.1	+	−
Inosine/methionine	6.8 ± 0.5	<0.1	+	−
Inosine/phenylalanine	7.5 ± 0.9	<0.1	+	−
Inosine/proline	8.9 ± 2.2	<0.1	+	−
Inosine/serine	10.5 ± 1.3	<0.1	+	+
Inosine/tryptophan (1 mM)	<0.1	<0.1	+	−
Inosine/tyrosine (1 mM)	0.8 ± 0.1	<0.1	+	−
Inosine/valine	5.8 ± 1.3	<0.1	+	−

[a] The average of triplicate experiments of two spore preparations is shown with 1 standard deviation.
[b] All amino acids are at 100 mM, except tyrosine and tryptophan, which are at 1 mM. Inosine is at 50 mM.
[c] Samples were done in triplicate and scored as germinating (+, >10% germination) or nongerminating (−, ≤10% germination) after 16 h.

TABLE 4. Effect of *gerS* mutation on dilute solution kinetics in *B. anthracis* ΔSterne 34F2

Germinant[b]	Maximum germination rate[a] (% germination/min)		Score for 16-h germination of mutant[c]	
	Parental	Δ*gerS*	Parental	Δ*gerS*
Alanine	<0.1	<0.1	+	+
Alanine/histidine	1.8 ± 1.6	<0.1	+	+
Alanine/proline	4.9 ± 1.9	<0.1	+	+
Alanine/tryptophan	1.8 ± 0.1	<0.1	+	+
Alanine/tyrosine	2.4 ± 0.7	<0.1	+	+
Inosine	<0.1	<0.1	−	−
Inosine/alanine	12.9 ± 0.9	<0.1	+	+
Inosine/cysteine	<0.1	<0.1	+	−
Inosine/histidine	6.0 ± 0.6	<0.1	+	−
Inosine/methionine	7.4 ± 0.9	<0.1	+	−
Inosine/phenylalanine	10.2 ± 0.4	<0.1	+	−
Inosine/proline	<0.1	<0.1	+	−
Inosine/serine	7.9 ± 1.5	<0.1	+	+
Inosine/tryptophan	4.6 ± 0.1	<0.1	+	−
Inosine/tyrosine	7.0 ± 0.4	<0.1	+	−
Inosine/valine	1.3 ± 0.7	<0.1	+	−

[a] The average of triplicate experiments of two spore preparations is shown with 1 standard deviation.
[b] All amino acids and inosine are at 1 mM.
[c] Samples were done in triplicate and scored as germinating (+, >10% germination) or nongerminating (−, ≤10% germination) after 16 h.

tested (1 mM each) to see if they could replace inosine. It was found that *B. anthracis*, like *B. cereus*, utilizes only the purine-based ribonucleosides, with inosine and adenosine being stronger germinants than guanosine in the presence of 1 mM serine. While inosine is not an independent germinant in *B. anthracis*, it is a strong cogerminant when coupled with amino acids, with several amino acids giving particularly strong germination responses even at a concentration of 1 mM. Percent germination for serine alone was 2.3 ± 0.2; for serine/inosine, 98.2 ± 0.6; for serine/adenosine, 97.4 ± 3.1; and for serine/guanosine, 27.0 ± 1.4 (values are the average of triplicate experiments of two independent samples after 30 min). The ribonucleosides, when tested alone, showed no germinative ability at any concentration. No significant germination was observed with 1 mM concentrations of cytidine, thymidine, adenine, ATP, ITP, ADP, and AMP. Throughout the remainder of this work these responses will be referred to as amino-acid-and-inosine-dependent (AAID) germination responses.

Influence of germinant concentration on germination kinetics. We evaluated the germinant solutions in two conditions, a high-concentration solution for each germinant (100 mM for most amino acids, 1 mM for tyrosine and tryptophan, and 50 mM for inosine) and a low-concentration solution with each germinant at 1 mM (Tables 3 and 4). There are several observations made from these studies. First, alanine (in the absence of secondary ligands) is only a strong germinant at high concentration, although there is some small degree of delayed germination inherent with 1 mM alanine at extended times (Table 4). The only rapid germination responses in dilute alanine were seen with the addition of a secondary amino acid (Table 4). Second, the AAID responses fall into two major groups when compared between concentration conditions. The first has G_is that drop as the concentrations of inosine and amino acid are lowered (AAID-1: L-alanine, L-cysteine, L-proline, L-serine, and L-valine). The second group has G_is that rise as the concentrations of inosine and amino acids are lowered (AAID-2: L-histidine, L-phenylalanine, L-tryptophan, and L-tyrosine) and will be discussed in more detail below (Tables 3 and 4). The response to L-methionine remains statistically the same between the two conditions and could constitute a third group. Though there are sufficient differences in the degree of change seen with each member of AAID-1 and AAID-2, evidence below supports these definitions.

Influence of pH. A germination-response curve related to pH was generated for the high concentration conditions of two representative strong germination solutions used earlier: alanine alone and methionine/inosine. Both stimuli showed significant germination over all physiologically relevant pH ranges. The alanine-alone curve shows a definite maximum (pH 8). It is of note that the other, more chemically complex condition does not. In fact, the maximum response of the methionine/inosine curve has a very broad plateau (pH 6 to >9) encompassing nearly the entire effective range (Fig. 1). This broadening of the pH curve into a plateau supports the hypothesis that germination solutions containing more than one germinant are triggering coordinated, overlapping cellular signals, especially since neither methionine nor inosine is an independent germinant. Multiple AAID-1 and AAID-2 germination responses were tested, and this pH range plateau was found to be a common property of all AAID responses (data not shown).

D-Alanine inhibition of strong germination responses. The amino acid enantiomer D-alanine is a potent inhibitor of the alanine germination response in most *Bacillus* species tested (13, 25, 26). When D-alanine was tested at various concentrations against the various germinants, it was found to have a

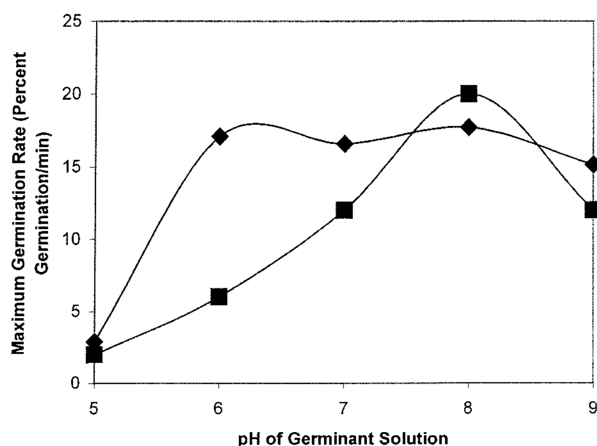

FIG. 1. pH response curves for alanine-alone and methionine/inosine germination solutions with *B. anthracis* ΔSterne 34F2. Amino acids are the L-isomers. The average of triplicate measurements is shown, although the standard deviation has been omitted for clarity (the standard deviation was $< \pm 4\%$ germination/min for all points). ■ is alanine (100 mM), ◆ is methionine/inosine (100 mM/50 mM).

mixed effect (Table 5). Even at a 1:1 ratio with L-alanine, D-alanine was found to completely inhibit the rapid alanine-alone and alanine/amino acid responses. However, the AAID responses required a high, 10:1, D-alanine-to-amino acid ratio to show appreciable effects. Even at the elevated concentration, D-alanine only completely inhibited the AAID-1 responses (Table 5), adding further evidence that there are at least four responses: alanine alone, alanine with a secondary amino acid, AAID-1, and the AAID-2 response.

***B. anthracis gerS* has homology to the *gerX* operon on pXO1 virulence plasmid.** Recent work has described a *gerA*-type operon on the pXO1 plasmid of *B. anthracis*. This operon, when eliminated by a deletion in the first coding region, results in attenuation with the mouse infection model for anthrax (5).

TABLE 5. Effects of D-alanine on germination of *B. anthracis* ΔSterne 34F2

Germinant (1 mM each)	Germination in 30 min[a] with:		
	No D-alanine	1 mM D-alanine	10 mM D-alanine
Alanine	+	−	−
Alanine/histidine	+	−	−
Alanine/proline	+	−	−
Alanine/tryptophan	+	−	−
Alanine/tyrosine	+	−	−
Inosine	−	−	−
Inosine/alanine	+	+	−
Inosine/cysteine	+	+	−
Inosine/histidine	+	+	+
Inosine/methionine	+	+	−
Inosine/phenylalanine	+	+	+
Inosine/proline	+	+	−
Inosine/serine	+	+	−
Inosine/tryptophan	+	+	+
Inosine/tyrosine	+	+	+
Inosine/valine	+	+	−

[a] Samples were done in triplicate and scored as germinating (+, >10% germination) or nongerminating (−, ≤10% germination) at 30 min. All amino acids are L-isomers unless stated. The buffer and reaction conditions are standard.

We used the sequence of *gerXA* to search the present unfinished genomic database at TIGR for a chromosomal germination locus. With the *gerXA* sequence, we found at least six prospective *gerA*-type sequences and then focused on the sequence containing the ortholog with the highest amino acid similarity to *gerXA* (31.7% identity, 52.1% similarity at the predicted amino acid level). Analysis of the data contained in contig 4752 of the unfinished sequence in the public database at TIGR shows a prospective *gerA*-type operon that we termed *gerS* for this study. The prospective operon is very similar to all other studied *gerA*-type operons and was chosen as a potential target for looking at chromosome-encoded germination responses.

Germination phenotype of Δ*gerSA* mutant strain. The *B. anthracis* ΔSterne Δ*gerSA* strain was surveyed against the entire panel of prospective germinants tested earlier (Tables 1 and 2). The mutant strain was not found to have any additional germination responses. However, the majority of the responses present in the parental strain were missing or attenuated in the mutant strain. Under the survey conditions only the L-alanine-alone, L-alanine/proline, and L-alanine/inosine responses were still detectable and strong. The complete loss of germination response was observed only in the solutions of the two AAID responses (Tables 3 and 4). All alanine/aromatic amino acid systems were returned to the alanine baseline level (Tables 3 and 4). The AAID responses were missing, except for the inosine/alanine response. In all dilute solutions the initial kinetic germination responses were below detectable levels. A number of the responses to the high-concentration solutions were also attenuated, though many could partially respond after 16 h (Tables 3 and 4). A pronounced microlag was observed in the alanine/aromatic amino acid germination responses and, to a lesser extent, in the alanine/proline response. It can be inferred from these data that the *gerS* locus is required in both AAID responses and may also play a role in the alanine/aromatic amino acid responses. Some loss of detection is inherent to the solutions containing L-alanine, L-serine, and L-cysteine, as they cause minor independent germination responses in the 16-h assay.

***gerS* locus is responsive to specific aromatic ring chemical structures.** Our data support the dual hypothesis that the *gerS* locus is involved in both the AAID-1/2 and the alanine/aromatic amino acid responses. This suggests that *gerS* may recognize similar chemical groups, since both the purine base of inosine (hypoxanthine) and the distinct functional groups of the amino acids tryptophan (indole), tyrosine (phenol), and histidine (imidazole) are all at least partially aromatic in nature. We hypothesize that the *gerS* locus may be responding to the aromatic component of these chemicals. To test this hypothesis, we measured the G_is of aromatic functional groups as distinct chemical entities paired with L-alanine (Table 6). While the rates with the aromatic subgroups were lower than those of their parent compounds, they were significantly higher than that of the alanine background. This phenotype was specifically eliminated in the *gerS*-null strain. The exception was with hypoxanthine, which was not functional as a purine base. Likewise, it is unknown why phenylalanine does not trigger a germination response when combined with alanine.

TABLE 6. Effect of aromatic side chains on germination in *B. anthracis*

Germinant[a]	Maximum G_i (% germination/min)[b]		Extent of germination after 15 min (% germination)[b]	
	Parental	Δ*gerS*	Parental	Δ*gerS*
10 mM alanine	1.4 ± 0.2	1.2 ± 0.4	6.5 ± 1.2	6.2 ± 1.0
10 mM alanine/1 mM tyrosine	6.9 ± 0.5*	1.6 ± 0.5	61.0 ± 5.6†	6.0 ± 0.8
10 mM alanine/1 mM phenol	2.9 ± 0.9*	1.7 ± 0.4	26.3 ± 3.6†	7.1 ± 0.9
10 mM alanine/1 mM tryptophan	4.7 ± 0.8*	1.9 ± 0.8	56.0 ± 5.4†	6.3 ± 2.0
10 mM alanine/1 mM indole	2.4 ± 0.3*	1.3 ± 1.0	33.2 ± 4.5†	7.5 ± 2.0
10 mM alanine/1 mM inosine	8.2 ± 0.9*	1.4 ± 0.6	73.0 ± 9.5†	10.5 ± 0.9
10 mM alanine/1 mM hypoxanthine	0.6 ± 0.2*	1.0 ± 0.2	6.0 ± 0.2	6.0 ± 0.5

[a] All amino acids are L-isomers; all solutions were done in the standard buffer.
[b] Results are the average of triplicate experiments on two independent preparations with 1 standard deviation. *, result is statistically different from the alanine baseline, $P < 0.01$. †, result is statistically different from that for the Δ*gerS* mutant, $P < 0.01$.

DISCUSSION

We investigated the role that amino acids and nucleosides play in the germination of *B. anthracis* ΔSterne in an attempt to define chromosome-encoded germination phenotypes. Combinations of amino acids and inosine were tested for their effect on germination kinetics and specificity. The studied germinants were described by maximum G_is, rate changes due to concentration effects, pH, and other parameters, and we focused on germination kinetics that were rapid (under 30 min) and strong (>50% germination). In contrast, germinant specificity was defined as an overall on/off phenotype response to a chemical entity. While it is possible to change the germination kinetics parameters within a discernible range through manipulation of the experimental system, the germinant specificity was a constant attribute that was unique to the system and was measurable as a germinated population even at prolonged time points.

Work with defined germinants led to only one strong and rapid independent germinant. L-Alanine (>10 mM) was the sole independently acting germinant (although serine and cysteine could act independently to a very small degree at very high concentrations and with prolonged incubation times). This is consistent with the data from a variety of *Bacillus* species that show high levels of L-alanine to be a strong germinant (3, 7, 10, 13, 15, 21, 23). The alanine germination response was inhibitable by the competitive enatiomer D-alanine at as little as a 1:1 ratio.

Assays to measure cooperative reactions between the amino acid L-alanine (1 mM) and the other amino acids registered at least two distinct types of responses. The first was an enhanced response of L-alanine when coupled with the aromatic amino acids, L-histidine, L-tyrosine, and L-tryptophan (the aromatic-enhanced-alanine [AEA] response). This response was shown to be attributable to the action of the aromatic side chains with L-alanine. The other response was governed by the response to L-alanine and L-proline. Additionally the Δ*gerSA* mutant showed this response to be different from the AEA response, though with comparable rates in germination kinetics profiles. Both of these responses were also inhibitable by 1:1 ratios of L-alanine:D-alanine.

Inosine is a strong independent germinant of *B. cereus* (1, 15) but not found to act as such in *B. anthracis* in these studies. Testing with *B. anthracis* showed two independent types of germinant specificities dependent upon inosine (AAID responses). The first, AAID-1, was accomplished through inosine and a nonaromatic amino acid. The second, AAID-2, was through inosine and an aromatic amino acid. These two germinant specificities exhibited differing germination kinetics. Inosine could be replaced only by other purine ribonucleosides, which is consistent with the work done with *B. cereus*.

The genetics of germination in *B. anthracis* are even less studied than the germination process itself. To date, besides our own work, there has only been one other paper to address a genetic basis of germination in *B. anthracis*. The work of Guidi-Rontani et al. (5) focused on genetically characterizing a *gerA*-type operon that is present on the pXO1 virulence plasmid and is designated *gerX*. The characterization of the *gerX* operon showed it to be a typical *gerA*-type tricistronic operon. Further work by the group showed some attenuation of *B. anthracis* in a mouse model upon disruption of this operon. However, attempts to match specific germinants to the action of the *gerX* locus were not attempted.

Using the *gerX* operon and particularly the *gerXA* ORF, we found the closest chromosomal match to be a putative operon that we designated *gerS*. It was important to find a chromosomal locus that controlled germination, since it has been shown that pXO1-negative and plasmidless strains of *B. anthracis* can germinate in animal models, indicating that in vivo germination is not fully attributable to a plasmid-borne locus. In addition, the *gerX*-null mutants (5) showed significant germination in the animal model, though the extent was impossible to determine due to experimental design limitations.

We constructed a null mutation of the *gerS* locus in a *B. anthracis* ΔSterne strain. This mutant was extensively tested, and two major conclusions can be reached. First, the *gerS* null mutations caused a loss of the kinetic enhancement seen in the AEA response, back to the alanine kinetic baseline. Second, the *gerS* mutation eliminated the germination specificity for AAID germination pathways. Further work showed that the *gerS* locus was an aromatic-responsive element that also plays an essential role in the AAID response, possibly through interactions with the aromatic purine ring of ribonucleosides.

Overall, the defined chemical germination data had a total of at least five distinct germination phenotypes: alanine alone (high concentration), alanine/proline, alanine/aromatic amino acids (AEA), AAID-1, and AAID-2. Using our data for *gerS* and the publicly available data for *B. cereus* from both MEDLINE and GenBank, we propose a tentative model for possible

germinant receptors to the individual germination phenotypes. These assignments are made with the assumption that multipartite signals could be recognized by multiple *gerA*-type receptors acting together, an idea originated with the *gerK*/*gerB* system in *B. subtilis* (10). Using this assumption, we can assign *gerS* as a component of our model, expanding out both the AEA and AAID responses to include the *gerS* locus.

A model of multiple germinants and receptors is consistent with the number of probable homologues found in a comparison of the *B. cereus* and *B. anthracis* genomes. Work is farther along on the genetics of *B. cereus* germination, and both the independent-alanine and independent-inosine responses have been localized to specific loci. The *B. cereus* independent-alanine response is localized to a *gerA*-type operon designated *gerL* (GenBank accession no. AF387344), which has a direct homologue in *B. anthracis* (unfinished; preliminary sequence data were obtained from http://www.tigr.org). This homologue could putatively be the alanine receptor for *B. anthracis* and part of the AEA response (along with the *gerS* locus), but direct experimental evidence is still lacking. The independent-inosine receptor in *B. cereus* is localized to a *gerA*-type operon designated *gerQ* (GenBank accession no. AY037930). Since *B. anthracis* does not have an independent-inosine response, it is not surprising that no homologue of *gerQ* is found in the *B. anthracis* genomic sequence data. However the alanine/inosine cogerminant response of *B. cereus* is controlled in part by a third *gerA*-type operon, *gerI* (1), which does have a direct homologue in *B. anthracis*. This homologue could serve as part of the AAID response element in our model (working in concert with the *gerS* locus and possibly *gerL*). Further work on the *B. anthracis gerI* homologue is currently being performed in our laboratory. An overview of the germination pathway model with putative *gerA*-type operon designations is presented in Fig. 2. Although all results reported in this paper are done in the plasmidless strain, the phenotypes and mutation were checked in the pXO1-bearing Sterne strain, with no significant differences being found (data not shown).

The elucidation of defined chemical germinants is the first step required to identify possible in vivo germinants for *B. anthracis* endospores, central to the infective cycle. We have shown a central and well-defined role for amino acids and purine ribonucleosides working in concert to effect rapid and extensive germination of *B. anthracis*, even at concentrations comparable to those present in some physiological samples. In addition we have described the first chromosomally encoded *gerA*-type operon in *B. anthracis* and have provided a probable chemical signal for it. That the signal for the *gerS* locus appears to involve aromatic rings is of importance, especially since early work on *B. anthracis* noted the phenomenon of phenolated endospores being more virulent in animal models (8). The continued selection of this microorganism in a host environment may have provided it with redundant mechanisms uniquely tuned to in vivo conditions. The germination response, activated first upon entering a host, is likely dependent on the chemicals presented. In turn, those germination responses appear to be tuned to best respond to multipartite signals, which could be advantageous in sensing a rich, host-like microenvironment.

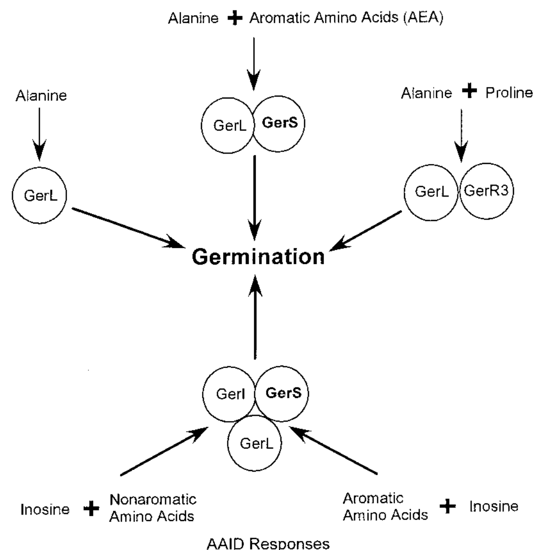

FIG. 2. Preliminary model of germination pathways in *B. anthracis* encompassing genetic data. These preliminary assignments were made with the assumption that multiple component signals could be recognized by multiple *gerA*-type receptors acting together, an idea originated with the *gerK*/*gerB* system in *B. subtilis*. The *gerI* and *gerL* designations represent the direct homologues in the *B. anthracis* genomic sequence of corresponding, characterized *B. cereus* germination operons.

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